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Award Number: W81XWH-07-1-0108

TITLE: Prostate Specific or Enriched Genes as Composite Biomarkers for Prostate Cancer

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REPORT DATE: March 2011

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
01-03-2011	Final	8 Jan 2007 - 7 Feb 2011

4. TITLE AND SUBTITLE

Prostate Specific or Enriched Genes as Composite Biomarkers for Prostate Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER

W81XWH-07-1-0108

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Institute for Systems Biology
Seattle, WA 96103

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

No abstract provided.

15. SUBJECT TERMS

No subject terms provided.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	10	19b. TELEPHONE NUMBER *(include area code)*

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INTRODUCTION:

Prostate-specific antigen (PSA) has been widely used as a single marker for prostate cancer diagnosis and screening. However, the high false negative and false positive rate coupled with over-diagnosis of clinically insignificant diseases have prompted search for better composite markers. The long-term goal of this study is to develop a panel of multiple protein biomarkers for prostate cancer diagnosis and stratification. Toward this end, we have orchestrated a concerted effort leveraging ISB's computational resource and proteomics expertise encompassing both antibody and mass spectrometry (MS) based technologies. To date, we have established a panel of >10 prostate-specific genes, and have embarked a two-pronged effort for validating these proteins in prostate cancer patient serum samples. On the one hand, we are experimenting antibody-based assays using an innovative technology—the nCounter platform which was originally developed at ISB and subsequently commercialized by NanoString Technologies—for multiplexed blood protein detection. On the other hand, we are actively developing targeted MS-based assays as part of a much grander proteomics effort characterizing tissue-specific proteins in the blood. We report here the latest progress on both efforts in the context of prostate cancer samples that we have collected over the past years.

BODY:

Development of ELISA assays for candidate prostate cancer biomarkers WDR19 and NDRG-1.

We have been working on developing a sandwich ELISA assays and single-antibody competition assays for **WDR19**. We found that the detection limits of the competition assays were not as low as those of the sandwich assays. It is very difficult to produce a recombinant form of the entire WDR19 protein. We were only able to express a truncated recombinant. With this truncated form, we achieved a dose response using a Rabbit Mab (RMab) and a Mouse Mab (MMab) in a sandwich assay. However we were not able to achieve a dose response using these two antibodies when blood was analyzed. Using two RMab (one biotin labeled), we were able to achieve a dose response from blood but the truncated recombinant failed to produce a dose response. We were also able to achieve dose response using the RMab's in various competition scenarios.

Two hundred thirteen serum samples have been screened with the sandwich immunoassay utilizing two rabbit Mabs (detector antibody labeled with biotin). This set was composed of serum from normal controls (N= 49), patients with benign prostatic hyperplasia or BPH (N= 44), cancer patients with gleason scores <=6 or primary prostate cancer (N= 68), and cancer patients with gleason scores >=7 or advanced prostate cancer (N=52). Since there is no available standard of known concentration for this assay, a sample previously determined to give a high signal was used to build a standard curve. 56% of the serum samples produced signals above L.O.D. (2.5 x background). The sandwich immunoassay was compared against two different types of competition immunoassays. Only 20% of the 213 samples produced signals above L.O.D in the competition assays. Also, there was no correlation between immunoassay values and western blots. This comparison was done on 35 different samples.

Receiver-operator-characteristics analysis was performed across all groups using the sandwich immunoassay values from both WDR19 and PSA. In all cases PSA outperformed WDR19 as a discriminating marker for prostate-cancer stratification.

NDRG-1 was initially discovered by our group to be disregulated in prostate cancer through an MPSS signature comparing mRNA from prostate cancer tumors to mRNA from normal prostate tissue. Based on sequence analysis, NDRG-1 was possibly a secreted protein, and thus a potential blood biomarker for prostate cancer. The goal was to develop a sandwich ELISA that could quantify NDRG-1 in blood and be useful as a clinical test for prostate cancer. Two commercial Abs against NDRG-1 were available at the time. These were tested for their potential as an Ab pair for the sandwich ELISA. After much effort we concluded that these two Abs were not compatible partners for a sandwich assay.

It had been reported in the literature that NDRG-1 was capable of binding Ni^{2+}, and so the potential to use Ni^{2+} as a capture agent in a sandwich ELISA was apparent. A working sandwich assay was achieved in this manner, but only worked with the purified antigen. Spiking increasing amounts of human serum into the assay decreased the signal in a proportional manner. The conclusion was that there was a substance in serum that bound NDRG-1 in such a way as to shield either the epitope or the Ni^{2+}-binding site. All efforts to develop a sandwich assay for NDRG-1 were halted at that point in an effort to pursue other, more promising biomarkers.

Given the difficulty and expense associated with developing ELISA assays for our biomarker candidates, we have decided to explore an alternative approach, which employs mass spectrometer operated in multiple reaction monitoring (MRM) mode, for validating new markers.

1. Targeted proteomics approach – Multiple Reaction Monitoring (MRM)

In recent years targeted proteomics using techniques such as multiple reaction monitoring (MRM) have been developed, allowing efficient and specific detection and quantification of potential protein markers in patient blood samples. MRM analysis is performed as tandem mass spectrometry via two or more stages. Typically, the first mass analyzer allows only a single selected mass (m/z of a peptide selected from a target protein) through and the third analyzer monitors for multiple user-defined fragment ions (transitions) produced by collision-induced dissociation in the second mass analyzer (collision cell). These techniques require one to have a predetermined set of protein biomarker candidates. In our previous report, we documented the identification of 14 genes—through a custom computational analysis of tissue transcriptome database—whose expression is highly enriched in the prostate tissue (Table 1, and last year's progress report). The benefit of MRM assays is that detection of multiple targets in blood no longer requires the complexities associated with ELISA development, so that marker validation time is no longer much of an issue.

However, detecting blood proteins has never been an easy task due to the great dynamic range of concentration of different proteins in plasma. These targeted techniques still require an initial reduction in the complexity of the serum. We have used Sigma-Aldrich's IgY-14 Spin Columns for this purpose. These columns contain antibodies against the 14 most abundant serum proteins that compose 95% of the total protein mass in blood.

Four sample groups have been chosen for the preliminary study. Pooled sets of serum from a randomized group of 10 individuals characterizing each sample group were prepared. The sample types are serum from patients with a Gleason score >=7 (advance prostate cancer), patients with a Gleason score <=6 (primary prostate cancer), patients with benign prostatic hyperplasia, and normal individuals. In addition, a pooled serum from advanced-cancer patients with the highest PSA levels was used as a control. Immunodepletion was conducted on these samples followed by MRM analysis via mass spectrometry.

From the 14 potential prostate cancer biomarker proteins, we have selected about 50 peptides and 300 transitions (Table 1). Method was built with calculated CE (collision energy) and duplicated MRM runs were performed as unscheduled analysis on an Agilent 6460 Triple Quad mass spectrometer. We have used this strategy to successfully detect many potential liver injury protein markers in the past. However, we were not able to detect any of the prostate protein targets in blood from the prostate patients in this pilot study. The most likely reason of failure might due to the fact that prostate is a very small organ and proteins secreted from prostate may present in much smaller amount as compared to large organs such as liver. To improve the possibility of successfully detection of prostate proteins in blood, in the frontend sample preparation aspect, we will try to use a more efficient depletion column: Seppro Human Super-mix from Sigma. Unlike the IgY 14 column used in the pilot study that depletes 14 top abundant proteins, Super-mix will deplete >30 most abundant proteins from samples and will, consequently, further enrich the target proteins in blood.

Table 1. Prostate-specific protein candidates and selected peptides for MRM validation.

Protein name	Peptide sequence
ACPP	FQELESETLK
ACPP	LSGLHGQDLFGIWSK
ACPP	ELSELSLLSLYGIHK
HOXB13	AAFADSSGQHPPDACAFR
HOXB13	ISAATSLSER
KLK2	NSQVWLGR
KLK2	HNLFEPEDTGQR
KLK3	HSQPWQVLVASR
KLK3	AVCGGVLVHPQWVLTAAHCIR
KLK3	LSEPAELTDAVK
KLK4	LDESVSESDTIR
KLK4	APCGQVGVPGVYTNLCK
SEMG1	LPSEFSQFPHGQK
SEMG1	HLGGSQQLLHNK
SEMG1	GISSQYSNTEER
SEMG1	DVSQSSIYSQTEEK
SEMG2	GHYQNVVDVR
SEMG2	GSISIQTEEQIHGK
SEMG2	DVSQSSISFQIEK
SLC45A3	NLGALLPR
SLC45A3	AEPGTEAR
SLC45A3	VVVGEPTEAR
SPDEF	AAAGAVGLER
SPDEF	WLLWTEHQYR
SPDEF	SPLGGDVLHAHLDIWK
SPDEF	IEDSAQVAR

STEAP2	EHYTSLWDLR
STEAP2	EIENLPLR
STEAP2	GPVVVAISLATFFFLYSFVR
STEAP2	DVIHPYAR
STEAP2	NQQSDFYK
STEAP2	IPIEIVNK
STEAP2	FYTPPNFVLALVLPSIVILGK
STEAP2	IILFLPCISR
TGM4	EVTVAVTSSPNAILGK
TGM4	SVTGFDSAHDTER
TGM4	NLTVDTYVNENGEK
TGM4	TSQIQGQVSEVTLTLDSK
TGM4	TYINSLAILDDEPVIR
TGM4	NTLAIPLTDVK
TMPS2	VLTQASNPVVCTQPK
TMPS2	LYGPNFILQVYSSQR
TMPS2	LNTSAGNVDIYK
TMPS2	LYHSDACSSK
TMPS2	VISHPNYDSK
TMPS2	NNIWWLIGDTSWGSGCAK

2. Development of multiplex protein detection system through the NanoString nCounter platform.

The NanoString nCounter system provides direct digital readout of the number of mRNA or DNA molecules in a small amount of sample, without the use of amplification. Current NanoString methodology involves mixing total sample RNA with pairs of capture and reporter probes, tailored to each mRNA. After hybridization and the washing away of excess probes, probe-bound target mRNAs are stretched on a surface and scanned to detect the fluorescent-barcodes of the reporter probes. This allows for up to 1000-plex measurement with high sensitivity and without amplification bias. The system works well with RNA from crude tissue lysates, flash frozen tissue, and formalin fixed paraffin embedded (FFPE) tissues, detecting as few as 0.5 transcripts per human cell. The system has also been shown to be particularly useful for biomarker measurement in cancer research. Payton et al. has used the nCounter to assay a large number of genes in primary clinical samples from acute myeloid leukemia patients. During the past year, we have modified the nCounter assays to allow for protein and for microRNA measurement. At ISB, we have used an approach similar to immunoPCR to convert protein measurement into DNA measurement. Briefly, the detection antibody of an ELISA pair is conjugated to an identifying 100-base DNA label. Sets of biotinylated capture and DNA detection antibodies are added to samples, allowed to bind, immunoprecipitated with streptavidin beads, washed, and the DNA labels are released. This allows for a highly-multiplexed ELISA-like assay to be performed on very small amounts of sample. Examples of both the RNA and the protein assays are shown in Figure 1. Additionally, NanoString has just introduced the Human miRNA Expression Assay Kit that enables researchers to profile more than 700 human and human-viral miRNAs with specificity and sensitivity comparable to qPCR,

but at a fraction of the cost. Yet more significant than the cost, it is the nCounter's efficient use of the limited sample material that makes it indispensible. The analysis of more than 700 nucleic acids by conventional means would require microgram quantities total RNA. The nCounter system will require only 100 ng, or on the order of 10,000 cells, a reasonable amount for biopsy samples. Thus, it is ideally suited for immediate clinical molecular lab applications. We are now experimenting the protein detection feature of the nCounter platform on the prostate-specific protein panels with good antibody pairs.

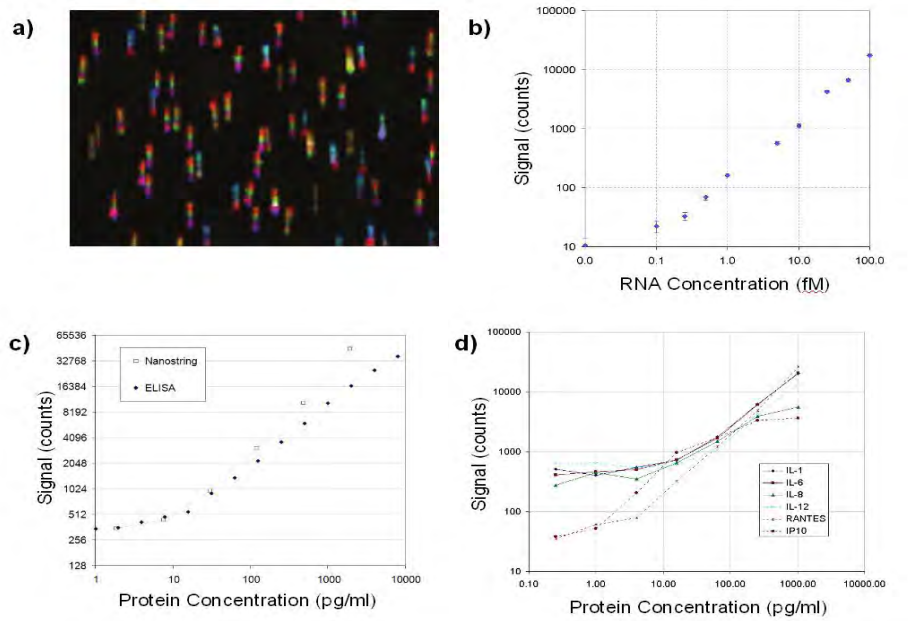

Figure 1. The Nanostring assay provides sensitive detection for transcriptomics and proteomics. (a) False-color image of immobilized reporter probes after an RNA assay. Each of the gene-specific reporters are identified by their unique sequence of fluorophores. (b) Excellent sensitivity and reproducibility is demonstrated with control targets spiked into a sample of 100 ng normal human RNA. Non-human controls were added at concentrations from 0 to 100 fM. (c) A calibration curve compares ISB's novel nCounter protein assay to a conventional ELISA using the same TNF-α antigen and antibodies. A similar lower limit of detection is observed. (d) The multiplex capability of the nCounter protein assay is demonstrated by the analysis of six cytokines using six pairs of DNA-labeled. Artificial samples were prepared by mixing various ratios of the protein standards in 10% serum/PBS

KEY RESEARCH ACCOMPLISHMENTS:

2011 Final Report

- Established a panel of 14 prostate-specific proteins
- Identified appropriate peptides and related transitions from each of the prostate-specific proteins to be tested by targeted MRM approach.
- Depleted top 14 abundant proteins from >40 prostate patients and control serum samples for MS validation.
- Developed multiplex protein-detection system using the Nanostring nCounter platform.

2010 Progress Report

- Developed a mouse antibody for TAGNL2.

- Established a novel panel of prostate-specific proteins to be tested by both antibody and MS based assays.

2009 Progress Report

- Developed a Sandwich immunoassay using a pair of monoclonal antibodies against WDR19, and screened more than 200 prostate cancer and control serum samples.
- Demonstrated that WDR19 is a not as good as PSA for prostate cancer diagnosis.
- Synthesized and characterized by MS 40 heavy isotopic labeled peptides from WDR19, TAGNL2, PSA, and other putative prostate cancer markers.

2008 Progress Report

- Demonstrated that WDR19 is a good tissue marker for prostate cancer prognosis.
- Generated and characterized good monoclonal antibodies for WDR19.
- Identified a novel prostate specific biomarker TAGNL 2

REPORTABLE OUTCOMES:

2011 Final Report

- Qiang Tian, Zhiyuan Hu, Shizhen Qin, James White, Danielle Yi, and Leroy Hood. Prostate-specific or enriched genes as composite biomarkers for prostate cancer. Innovative Minds in Prostate Cancer Today (IMPaCT), Orlando Florida, March 9-12, 2011

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CONCLUSION AND FUTURE PLANS:

PSA is currently the best blood biomarker for prostate cancer; however, it lacks the ability to distinguish between primary prostate cancer and benign prostatic hyperplasia. PSA also

provides little information as to how the disease might progress. Better techniques for the diagnosis and prognosis of prostate cancer are needed.

Future studies will involve using the SuperMix column from Sigma-Aldrich which depletes serum even further. It would be advantageous to collect proximal prostate serum before it is diluted into the full serum of the body. Identifying targets at proximal sites may help us zero in on targets that could possibly be observed in peripheral serum. From the mass spectrometry perspective, we will try to optimize the MRM conditions by using the Agilent OPTIMIZER software to perform automated compound specific optimization for each peptide's fragmentor and collision energy to achieve maximum sensitivity, hence to improve the chance of detection in the next phase of study. As more antibody pairs of the prostate-specific protein panel become available, they will be adopted to the Nanostring nCounter platform for further validation.

REFERENCE:

None.

APPENDICES:

None.